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ORIGINAL ARTICLE

Antimicrobial Resistance of Secondary Bacterial Infections in Patients Hospitalized with Covid-19

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is an emerging serious global health problem**.** It has been recognised for a considerable time-period, that viral respiratory infections predispose patients to bacterial infections, and that these co-infections have a worse outcome than either infection on its own.

This study was carried out on 100 samples of sputum from COVID-19 patients. During the laboratory diagnosis, 156 bacterial isolates were obtained from the positive samples . The Gram-positive bacteria isolates included Strptococcus pneumonia 64(40%) Streptococcus pyogenes, 7 (4%), Streptococcus mitis 1 (1%) Streptococcus mutus 1(1%) , Streptococcus parasanguinis 1(1%) Staphylococcus eidermidis 10(6%), staphylococcus aureus 4 (2%), Micrococcus lutus,1 (1%). Whereas, Gram-negtive bacteria included Pseudomonas aeruginosa 9 (6%), E coli 10 (6%), Serratia marcescens, 3 (2%), Klebsiella pneumonia 31 (19%), H.influenzae 10 (6%) Acinetobacter baumannii , 4 (2%) .The isolates varied in their response against the antibiotics ; and Gram positive bacteria were significantly ($p<0.05$) more sensitive to the antibiotic then Gram negative. To detect the Streptococcus pneumonia isolates , the house keeping Eno genes was screened. Results showed that all the isolates, had Eno gene (100%). Furthermore, This study was carried out in order to detect tet-L and ermB gene in 10 S.pneumonia isolates . genes were Results showed that all the isolates, numbering 10, had both tetL and ermB genes(100%).

Keywords : Bacteria, Co-infections, COVID-19, Eno gene, tet-L gene , ermB gene

INTRODUCTION

Coronavirus disease is a new public health crisis threatening the humans. This pandemic disease is caused by the novel betacoronavirus. Covid-19 has very important clinical features such as high rates of transmission, mild to moderate clinical manifestation. with more serious radiological abnormalities seen in the elderly. Remarkably Coronaviruses enter cells through the ACE-2 receptor (Zhoug et al., 2020**).**

Coronaviruses enter cells through the ACE-2 receptor. Sin-converting enzyme 2 (ACE2) receptors to invade human cells, and these receptors are highly expressed in the intestinal epithelium (Ni, W, 2020).

Coronaviruses are members of the Nidovirales order's Coronaviridae family (Su et al. 2016). There are four genera that have been identified: alpha, beta , gamma , and delta . (Perlman and Netland 2009).

Coronavirus is one of the most common pathogens that primarily targets the human respiratory system SARS-CoV-2 infection.

The asymptomatic with coronavirus disease are range of respiratory symptoms, including fever, dry cough, and dyspnea, as well as pneumonia, pulmonary edema, acute respiratory distress syndrome, and multiple organ failures, necessitating hospitalization in an intensive care unit and, in severe cases, death. (Chen N et al., 2020).

COVID-19 patients had higher levels of opportunistic pathogens (OPs), a type of commensal microbiota that can becomes pathogenic when dysbiosis or the host's immune system is damaged (immunocompromised host)..(Brown SP et al., 2012) **.** When compared to healthy persons, COVID-19 patients had significant changes in their gut flora, which is consistent with earlier studies on respiratory viral infections (Zuo T et al., 2021). The altered composition of the human microbiome could affect the body's ability to fight inflammation and regulate the immune system, which could be harmful in the case of SARS-CoV-2 infection. (. Novakovic M., 2020 **)**.

Antibiotics such as amoxicillin, azithromycin, and clavulanate have been used in combination to treat COVID-19 patients with pneumonia who are receiving non-intensive care since a substantial percentage of patients are acquiring secondary infections (Pelfrene et al., 2021). The World Health Organization proclaimed the global problem of antibiotic resistance, a worldwide hazard to public health, in 2014, implying that this vital defense was decreasing. Antibiotics are required in the majority of severe COVID-19 patients, according to several studies (Martinez-Guerra et al., 2021).

Bacteria may manifest natural and acquired resistance to antibacterial drugs through variety of mechanisms: reduction of membrane permeability to antibiotics; drug inactivation/modification; efflux of antibiotics; and modification of cellular target(s) (Surette and Wright, 2017). Resistance mechanism of various antibiotics is described in .Tetracycline are broad-spectrum antibiotics used in the treatment and prevention of bacterial infections (Ardic N et al .,2005). Most tetracycline resistant bacteria have acquired tetracycline resistance genes (tet). tetL encodes resistance antibiotic tetracycline , this gene was discovered in different types of gram positive bacteria such as Bacllius, Staphylococcus, Streptococcus ,Enterococcus (Frazon et al., 2010).

Development of penicillin resistance in the pneumococcus in the 1980s–1990s shifted antibiotic treatment of suspected pneumococcal upper respiratory infections and pneumonia to macrolides. Widespread macrolide use, however, is associated with increased macrolide resistance in S. pneumoniae (Malhotra-Kumar et al., 2007).

MATERIAL AND METHOD

Samples Collection : Sputum samples were collected from 100 Covid -19 patients (both gender). Samples collection, isolation, identification, and antibiotic sensitivity test were all part of the first stage. Second stage includes genetic analysis using PCR to identify isolates that are more prevalent (Table 1).

Susceptibility test: The susceptibility of the isolates to selected antibiotics was tested according to the guides of CLSI (2021) using Kirby-Bauer method and as follows (Vandepitte et al., 2003). Three to five colonies were grown on nutrient agar and transported to tubes contain normal saline till the turbidity of MacFarland's standard (1.5x108 cell/mL) was achieved. A sterile cotton swab was inserted into a tube containing bacterial suspension then it was rotated around and pressed against the inner walls of the tube to remove excess feed, then spread across petri dishes containing Muller-Hinton agar, in different directions to ensure a homogenous growth. Antibiotic disks used in the study mentioned in table 3.6 were placed on the surface of the cultured media and with equal distance from each other and were pressed in using sterile forceps then stored at 37 °C for 24 hours. Results were read by measuring

the diameter of the inhibition zone around the antibiotic disks then comparing them to the standards mentioned in CLSI, 2021.

Extraction of genomic DNA: The genomic DNA was extracted from 10 isolates of S. pneumonia by DNA extraction kit(ZymoBIOMICS DNA Kits) following the manufactures instructions DNA extracts were prepared from 10 S. pneumonia isolates of based on their antibiotic resistance (MDR) .DNA concentration was between 15 30ng/µL. Whereas the purity of DNA was found to between 1.6 1.8.

Polymerase Chain Reaction Technique (PCR): Components of each PCR mixture were mixed together in Eppendorf tube by vortex before settings into thermocycler.The reaction was performed in a PCR thermal cycler apparatus, and after several trials, and according to the manufacture's guide (Table 1,2,and3).

Table 1: The primers used in this study

NO	Primer Name	Sequences (5/ - 3/)		Gene	Amplicon size (bp)
2	Eno	F	GACGGTACTCCTAA CAAAGGTAAA	Eno	110
		R	ATAGCTGTAAAGTG GGATTTCAAG		
3	tetL	F	AAATTGTTTCGGGT CGGTAA	TetL	537
		R	ATTCCCCCACAAAG AACTCC		
4	ermB	F	CCTTTACGAAATTG GAACAGGTAAAGGC	ermB	360

This technique accorded with all primers in this study, as listed in table**2**

Table 2: Protocol of PCR reaction mixture volumes used in the current study

Components	Concentration
Taq PCR PreMix	5µl
Forward primer	10 picomols/µl (1 µl)
Reverse primer	10 picomols/µl (1 µl)
DNA	1.5µl
Distill water	16.5 µl
Final volume	25µl

Table 3: Programs of PCR thermocycling conditions for genes detection

PCR Program	Tm (°C)	Time	No. of cycle
Initial Denaturation	94°C	3 min.	1 cycle
Denaturation -2	94°C	45sec	35 cycle
Annealing	40,45,47,49,51,53,5 5 °C	45sec	
Extension-1	72°C	45sec	
Extension -2	72°C	7 min	1 cycle
Hold	10°C	10min	1 cycle

RESULTS AND DISCUSSION

Description of study samples: This study performed from October- 2021 to January -2022. It has carried out by using 100 sample covid -19 patients that were Baequba teaching hospital, from both sex with age range (15–80) years.

Bacterial Culture: Bacteria isolates were identified depending on their physical characteristics and cultuer on blood, MacConkey ,chocolate agar .The results culture of sputum sample to 100 Covid -19 patient shows, During the laboratory diagnosis, 156 Bacterial isolates are obtained from the positive samples and negative bacteria .

The isolates of Gram-positive bacteria were Streptococcus pneumonia 64(40%) , Strptococcus pyogenes, 7 (4%), Strptococcus mitis 1 (1%) Streptococcus mutus 1(1%) , Strptococcus parasanguinis 1(1%) Staphylococcus eidermidis 10(6%), staphylococcus aureus 4 (2%), Micro coccus lutus,1 (1%). The isolates of Gram-negtive bacteria , which included Pseudomonas aeruginosa 9 (6%), E. coli 10 (6%) Serratia marcescens, 3 (2%), Klebsiella pneumonia 31 (19%) H.influenzae 10 (6%) Acinetobacter baumannii , 4 (2%) .

Susceptibility test: The sensitivity test was performed against Gram-positive bacteria as shown in Table 4 and Fig 1. It is clear that, the isolates of Staphylococcus aureus showed the highest resistance (67%)to all selected antibiotics, followed by Streptococcus mutus and Strptococcus pneumoniae that presented around 58%of resistance to all antibiotics. Whereas, the isolates of Streptococcus mitis could resist only half of the antibiotics (50%). Both of Micrococcus luteus and Strptococcus parasanguinis isolates were able to resist 25% of the antibiotics (Table 4 and Fig1).

Hoverer, there is no significant (p value =0.08) between the total resistance and sensitivity to all antibiotics (Table 2). It is clear from all bacterial isolates were sensitive to Meropenem (100%); whereas, 83 % of bacterial isolates were sensitive to Ceftriaxone , Doxycycline, Imipenem, and Vancomycin; Half of selected isolates (50%) displayed resistance against Minocycline and Ofloxacin. Whereas, around third (33%) of bacterial isolates were sensitive to Penicillin and Trimethoprime .

And finally, only 17 % of the selected isolates did not resist the Azithromycin. On the other hand, all the selected isolates resisted both of Amoxicillin and Cefeipime (Table 4). Penicillins and cephalosporins. Compounds are “bacteriostatic” when it inhibits growth, allowing the host immune system to eliminate the bacterial cell e.g .sulfonamides, tetracyclines, or chloramphenicol (Guardabassi and Courvalin, 2006). Antibiotics affect microbes via diverse mechanisms of action, such as (i) inhibition of cellular structure or metabolic processes, (ii) inhibition of bacterial cell wall synthesis, (iii) interference with cell membrane functions, (iv) inhibition of bacterial protein synthesis, and (v) inhibition of nucleic acid synthesis.

Table 4: Antibiotic sensitivity/ resistance pattern of clinical bacteria (G^+Ve) toward 13 antimicrobial agents (%).

Anitbiotics	Micrococcus luteus	Staph aureus	Strp. pneumonia	Strp. mitis	Strpt. mutus	Strpt. Parasanguinis	Resistance %	Sensitive%
Meropenem	S	S	S	S	S	S	0	100
Ceftriaxone	S	S	S	S	R	S	17	83
Doxycycline	S	R	S	S	S	S	17	83
Imipenem	S	S	R	S	S	S	17	83
Vancomycine	S	R	S	S	S	S	17	83
Minocycline	S	R	R	S	R	S	50	50
Ofloxacin	R	S	R	R	S	S	50	50
Penicillin	S	R	R	R	R	S	67	33
Trimethoprime	S	R	R	R	R	S	67	33
Azithromycin	S	R	R	R	R	R	83	17
Amoxicillin	R	R	R	R	R	R	100	0
Cefeipime	R	R	R	R	R	R	100	0
P-value=0.08								
0.5	25	67		58	50	58	25	Resistance %
	75	33		42	50	42	75	Sensitive%

* The antimicrobial susceptibility pattern of isolated bacteria based the criteria of Laboratory Standards Institute [CLSI, 2014] .R resistance; S sensitive

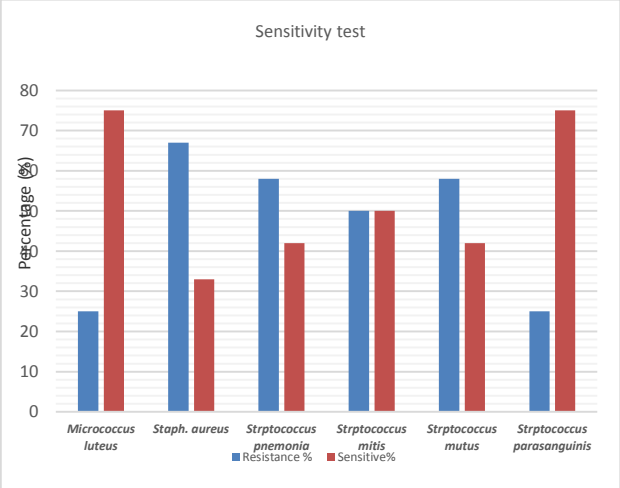

Figure1: Multidrug resistance pattern (%)of bacterial pathogens (Gram +Ve) isolated from 100 patients with Covid-19

Gram-negative bacteria: The sensitivity test was performed against Gram-negative. It is clear from Table 5- and Fig 2 , there is a significant ($p<0.01$) differences between the total resistance of bacterial cells to all antibiotics (Table 4). The isolates of P. aeruginosa and E.coli showed the highest resistance (85 %)to all selected antibiotics, followed by H .influenzae isolates that presented around 77% of resistance .Whereas, the isolates of Klebsiella pneumonia resisted 69% of antibiotics. Serratia marcescenns isolates were able to resist 68% of the antibiotics, and finally Acintobacter baumannii isolates resisted less than half (46 %) of the antibiotics (Table 5 and Fig2). Bacterial isolates were sensitive to 83% of Amoxicillin (Clavulanic acid) and Imipenem, Polymyxin .

Whereas, 33 % of bacterial isolates were sensitive to Trimethoprime, Ofloxacin, Azithromycin ,and Tobramycin. Lastly, only 17% of bacterial isolates were sensitive Ceftazidime , Ceftriaxone , Doxycycline, and Tigecycline. However, all the selected isolates (100%) resisted both of Pipracillin and Cefeipime (Table 5).

Table 5: Antibiotic sensitivity/ resistance pattern of clinical bacteria (G-Ve) toward 13 antimicrobial agents (%).

Bacterial isolate	H .influenzae	Serratia marcescenns	P.aeruginosa	Klebsiella pneumonia	E.coli	Acintobacter baumannii	Sensitive %	Resistance %
Trimthaprine	R	S	R	S	R	R	33	67
AmoxicillinClavulanic acid	S	R	R	R	R	R	83	17
Azithromycin	R	R	S	R	R	S	33	67
Cefeipime	R	R	R	R	R	R	0	100
Ceftazidime	R	S	R	R	R	R	17	83
Ceftriaxone	R	S	R	R	R	R	17	83
Doxycycline	R	R	R	R	R	S	17	83
Imipenem	S	S	R	S	S	S	83	17
Ofloxacin	R	R	R	S	R	S	33	67
Pipracillin	R	R	R	R	R	R	0	100
Polymyxin	S	R	S	S	S	S	83	17
Tigecycline	R	R	R	R	R	S	17	83
Tobramycin	R	S	R	R	R	S	33	67
P value	0.02*							
***0.01**	77	62	85	69	85	46	Resistance %	
	33	38	15	31	15	54	Sensitive%	

*The antimicrobial susceptibility pattern of isolated bacteria based the criteria of Laboratory Standards Institute [CLSI, 2014] .R resistance; S sensitive

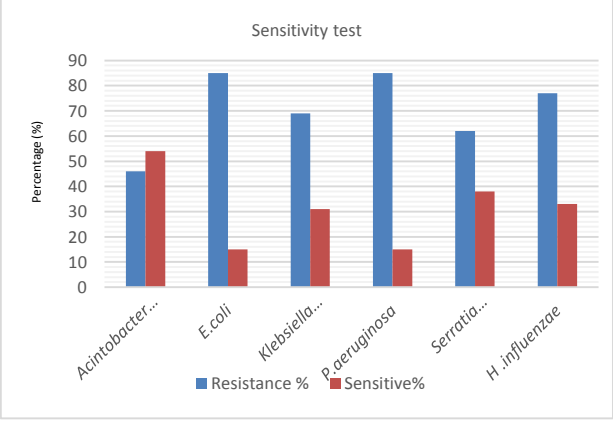

Figure 2: Multidrug resistance pattern (%)of bacterial pathogens (Gram -Ve) isolated from 100 patients with Covid-19 at Baquba city.

Molecular Detection

Molecular detection of Eno: This study was carried out in order to detect Eno gene in 10 S.pneumonia isolates. Eno genes were screened by PCR technique was used to detect the S.pneumonia species (measuring 110 bp). Results showed that all the isolates, numbering 10, had Eno(100%)

These were in agreement with the study (Sakai, F et al 2013) of who found that all (100%) of isolates S.pneumonia was positive for Eno gene

in Figure 3 The results of gel electrophoresis for PCR product by using specific primers

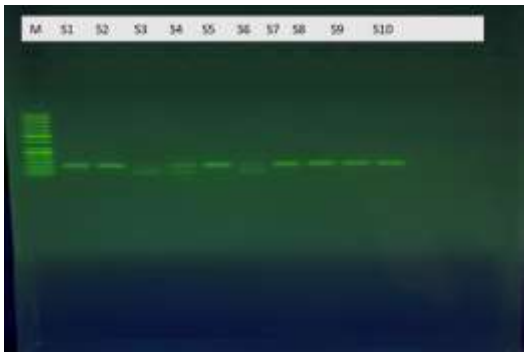

Figure 3: PCR product the band size 110 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

Molecular detection of tetL gen: This study was carried out in order to detect tet-L gene in 10 S.pneumonia isolates .tet-L gene

were screened by PCR technique was used to detect the genes of tet-L measuring 537 bp. Results showed that all the isolates, numbering 10,
had tetL(100%) for isolates. . as show in Figure (4)

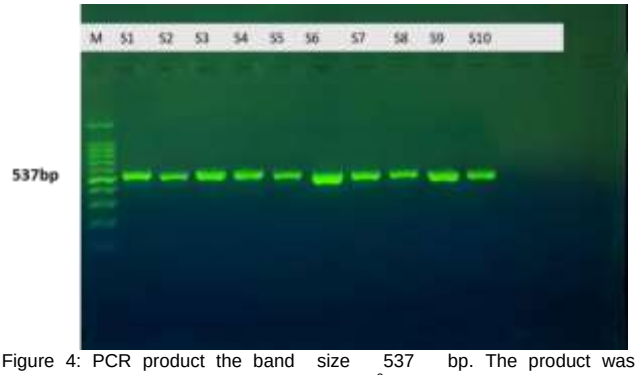

Figure 4: PCR product the band size 537 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

Molecular detection of ermB gene: This study was carried out in order to detect ermB gene in 10 S.pneumonia isolates.ermB gene were screened by PCR technique was used to detect the gene of tet-L measuring 360 bp. Results showed that all the isolates, numbering 10, had ermB (100%) for isolates as show in Figure 5 This result close with the study Ayadi, B. M et al .,2020(PCR showed that 78 isolates (90.7%) carried the ermB gene.

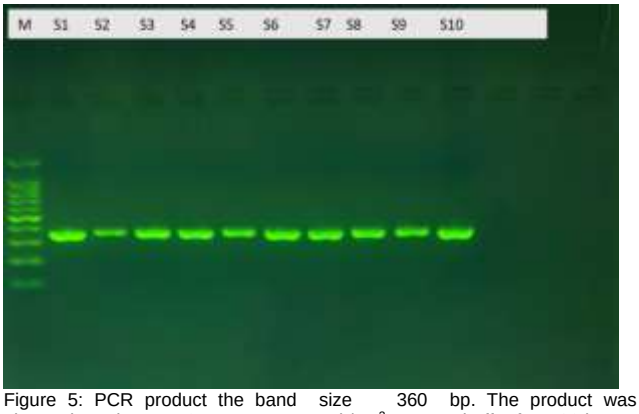

Figure 5: PCR product the band size 360 bp. The product was electrophoresis on 1.5% agarose at 5 volt/cm^2. 1x TBE buffer for 1:30 hours. N: DNA ladder (100).

In conclusion patients with COVID-19 receive antibiotic treatment either in the outpatient or inpatient setting .There are two main reasons that patients with COVID-19 potentially receive antibiotic therapy. First, COVID-19 symptoms may be similar to bacterial pneumonia. Second, patients with COVID-19 may acquire a secondary bacterial infection that requires antibiotic treatment . Although antibiotic resistance is a common phenomenon, antibiotic consumption is a leading cause of the emergence of antibiotic resistance as well as of the acquisition, development, and spread of the resistome.

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